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THE SUPRACHIASMATIC NUCLEUS IS A FUNCTIONALLY HETEROGENEOUS TIMEKEEPING ORGAN

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Abstract

Ever since the locus of the brain clock in the suprachiasmatic nucleus (SCN) was first described, methods available have both enabled and encumbered our understanding of its nature at the level of the cell, the tissue and the animal. A combination of *in vitro* and *in vivo* approaches has shown that the SCN is a complex heterogeneous neuronal network. The nucleus is comprised of cells that are retinorecipient and reset by photic input; those that are reset by non-photic inputs; slave oscillators that are rhythmic only in the presence of the retinohypothalamic tract; endogenously rhythmic cells, with diverse period, phase and amplitude responses; and cells that do not oscillate, at least on some measures. Network aspects of SCN organization are currently being revealed, but mapping these properties onto cellular characteristics of electrical responses and patterns of gene expression are in early stages. While previous mathematical models focused on properties of uniform coupled oscillators, newer models of the SCN as a brain clock now incorporate oscillator and gated, non-oscillator elements.

The Brain's Clock as a Construct

The function of the suprachiasmatic nucleus (SCN) was discovered during the era that identified the hypothalamus as the site of several brain “centers” governing homeostatically-regulated behaviors. Ablation of the lateral hypothalamus resulted in a significant reduction of eating behavior and weight loss, hence an eating center. Destruction of the ventromedial hypothalamus produced obesity, hence a satiety center. The opportunity to electrically self stimulate the brain was so powerful a reward that all other motivated behaviors were put aside, hence pleasure centers. And lesions of the SCN led to dramatic behavioral and physiological arrhythmicity, hence a timekeeping center.

Today, most of these center constructs have fallen out of favor. For the eating center, it was realized that “specific” hypothalamic lesions damaged fibers of passage, that lesioned animals showed sensory neglect and no longer responded to afferent inputs, and that other, major consequences accompanied weight loss in addition to disrupted feeding. For the pleasure centers, once the neural network and transmitter systems involved in self stimulation were delineated, it was obvious that no single center controlled the behavior. The notion of brain centers began to lose its heuristic value, and the centrist view was even labeled a “millstone” rather than a “milestone” to progress in neurobiology (Coscina, 1976).

For the SCN, such a revision has not occurred. The body of evidence identifying the nucleus as the master circadian pacemaker in mammals is multi-disciplinary in nature, and the strength of this functional localization is unsurpassed by that of any other structure in the vertebrate

brain (for review, see Klein et al., 1991). Lesions of the SCN result in a breakdown of the generation or entrainment of a wide array of rhythms; and they never recover, no matter how early in development ablation is performed. The SCN's circadian oscillation is seen *in vivo* and *in vitro*, using metabolic, electrophysiological, and molecular assays, and electrical or pharmacological stimulation causes predictable phase shifts of these rhythms. Neural grafts of fetal SCN tissue re-establish overt rhythmicity in arrhythmic, SCN-lesioned recipients, and the rhythms restored by the transplants display properties characteristic of the circadian pacemakers of the donors rather than those of the hosts. Thus, among the hypothalamic "centers" of decades ago, the SCN as timing center has retained its conceptual value.

What we are learning now is that the physical center is not an indivisible homogeneous cellular syncytium but a complex heterogeneous neuronal network with intra-center localization and specialization of function. In this review, we highlight how the locally-distributed network properties within the SCN are key to its pacemaker function. It is only through the combined use of advanced morphological, physiological, molecular, and genetic tools that researchers have begun to delineate the functional compartmentalization of this tissue that acts as the brain's circadian clock.

In the Beginning: Anatomical Heterogeneity but Functional Homogeneity

The decade of the 1970s is remembered as the time during which the SCN was implicated as the site of a mammalian circadian pacemaker. In 1972, lesions of the rat SCN were reported to abolish circadian rhythms of behavioral (wheel running and drinking) (Stephan and Zucker, 1972) and endocrine (corticosterone) (Moore and Eichler, 1972) activity, and later in the decade, endogenous rhythms of SCN glucose utilization (measured by ^{14}C-labeled deoxyglucose uptake) (Schwartz and Gainer, 1977) and electrical activity (recorded as the overall firing rate of multiple neurons) (Inouye and Kawamura, 1979) were demonstrated in intact rats. It was recognized even then, and certainly by 1980, that SCN cells were not a homogeneous population. Nissl and silver stains, Golgi impregnations, and electron microscopy of the rat SCN revealed two predominant subdivisions: cells in the dorsomedial part of the nucleus were smaller and more tightly packed than those in the ventrolateral part (van den Pol, 1980). This dorsal / ventral distinction corresponded to the ventrolateral segregation of most SCN inputs (from the retina, raphe, and lateral geniculate), and it was recapitulated by immunohistochemical identification of peptides in SCN cell bodies (e.g., arginine vasopressin (VP) dorsomedially and vasoactive intestinal polypeptide (VIP) ventrolaterally) (Inouye and Shibata, 1994; Moore et al., 2002).

Despite this morphological evidence for regional compartments within the SCN, physiological data seemed to favor equipotentiality across the SCN, without a clear localization of function. Small, partial electrolytic lesions were made in an effort to determine if specific regions might govern different rhythms (van den Pol and Powley, 1979; Pickard and Turek, 1985), but this approach, in the absence of markers for specific cell phenotypes and assessment of destruction of passing axons, could not characterize the critical factor in the observed dysrhythmias. What was generally found was a correlation between the volume of SCN destroyed – without regard to the unilaterality or regionality of the damage – and a shortened free-running period of the wheel-running rhythm, prompting the view that "SCN tissue is homogeneous in its contribution to rhythmicity even though the anatomical and biochemical heterogeneity of the nucleus in rats would suggest otherwise" (Davis and Gorski, 1984). Supporting this view were the discoveries that circadian oscillation of the fetal SCN antedated its regional specialization (Reppert and Schwartz, 1984) and in the adult, that the intranuclear distribution of ^{14}C-labeled deoxyglucose uptake (Schwartz et al., 1987) and multiunit electrical discharge activity (Bouskila and Dudek, 1993) showed no obvious dorsal / ventral difference.

Reducing SCN Tissue to Cells and Slices *in Vitro*

In 1995, individual dissociated SCN neurons were shown to oscillate independently with different circadian periods *in vitro* (Welsh et al., 1995), demonstrating that a circadian clock was localized within individual cells rather than arising as an emergent property of an SCN network. The results were interpreted to suggest that the oscillatory capacity could not be restricted to any particular subset of neuropeptide-containing cells; altogether, immunochemically-identified cells accounted for only 23% of all the neurons in culture, whereas 50% of all the neurons were rhythmic. The authors suggested that their data were consistent with the possibility that the non-oscillating cells in culture were actually from outside the SCN and that all SCN neurons were functioning "clock" cells. The cellular homogeneity implied by this interpretation stimulated models for how such cells might be synchronized within a coupled network (Liu et al., 1997).

In order to study SCN cells over multiple circadian cycles *in vitro*, but with a model that would preserve an *in vivo*-like dorsal / ventral architecture, hypothalamic slices containing the SCN have been incubated for weeks (either embedded in plasma clots on coverslips in rotating roller tubes or adhered to filters in stationary cultures). Such "organotypic slice cultures" lost at least 70% of their neurons and flattened to a few cell layers thick, but they survived, expressed immunoreactive AVP and VIP regionally as *in vivo* (Tominaga et al., 1994; Belenky et al., 1996), and exhibited circadian rhythms of the release of both peptides into the medium. When the cultures were treated with antimitotics, the two peptide rhythms appeared to free-run separately with different circadian periods (Shinohara et al., 1995), suggesting that they represented a chemical manifestation of comparable, equipotent oscillators in both the dorsal and ventral SCN subdivisions. A later study of such rat slice cultures, recording multi-channel electrical activity along with rhythms of AVP and VIP release, confirmed oscillations in both the dorsal and ventral SCN, although they did report very subtle dorsal / ventral differences, including evidence for a less stable VIP than AVP rhythm and a lower proportion of ventrolateral than dorsomedial neurons with rhythmic firing rates (Nakamura et al., 2001). Most recently, organotypic slices have been made from neonatal transgenic mice expressing a luciferase reporter driven by an oscillating clock gene promoter (*mPer1*) (Yamaguchi et al., 2003). Irrespective of location, virtually all of the luminescent cells (99.2% out of a total of 1177) exhibited circadian rhythmicity, although the proportion of total SCN cells that were luminescent was not described. Details of slice preparation, thickness, culturing methods, species of origin and measures used to assay rhythmicity may lead to variable results.

That cultured slices might be an incomplete model of SCN tissue organization is perhaps not too surprising, as they likely undergo some degree of reorganization. For example, although AVP and VIP are present "organotypically" in cultured slices, gastrin releasing peptide (GRP; a prominent ventrolateral neurotransmitter) expression at the adult level is lost (Wray et al., 1993). Importantly, the apparent functional redundancy in cell and slice cultures contrasts with data obtained from acutely-prepared slices. As early as 1984, it was reported that the circadian rhythm of spontaneous single-unit discharge rates in the ventrolateral, but not the dorsomedial, SCN was abolished in slices made from rats housed in constant darkness (DD) or bilaterally enucleated (Shibata et al., 1984). Studies of this kind have generally been performed by sampling the extracellular spike activity of single units for short time intervals at different phases across the circadian cycle, pooling the data from a number of slices and phases, and then determining the phase of peak firing rate for the population of units as a whole. More recently, acute slices made from neonatal transgenic mice expressing a short-half-life green fluorescent protein (GFP) reporter driven by the *mPer1* promoter (Quintero et al., 2003) indicate that in slices made from mice housed in a light-dark cycle (LD), 11% of the imaged cells exhibited nonrhythmic *Per1*::GFP expression; from mice housed in DD, this proportion increased to 26%. Rhythmic cells in LD slices were more likely ventral than dorsal (64% vs

36%, respectively), whereas the opposite distribution was found in DD slices (43% vs 57%). While these methods suggested functional heterogeneity among SCN cells, they did not associate function with phenotype and left unexplained the differences among experimental results.

SCN Tissue Organization and Heterogeneous Gene Expression

In addition to cells and slices *in vitro*, SCN tissue must be studied *in vivo* to learn how it actually functions in intact animals. This goal was invigorated in the 1990s by the identification of putative "clock" genes, molecules that appear to lie at the clock's core oscillatory mechanism as two interacting feedback loops (for review, see Van Gelder et al., 2003). In one loop, transcription of the *Period* (*Per*), *Cryptochrome*, and possibly *Timeless* genes is negatively regulated by their protein products, which inhibit (with a time delay) the DNA-binding activity of the positive bHLH transcription factors Clock and Bmal1 / Mop3 (in mammals). Within this loop, the essential time delay is provided by a phosphorylation-dependent variation in the stability of the Per protein (mediated by casein kinase 1 epsilon) and its complex with Cryptochrome. In the second loop, *Bmal1 / Mop3* mRNA and protein oscillate (in anti-phase to *Per*) by driving circadian expression of the *Clock* repressor, Rev-erb-alpha.

In the SCN, the most extensively studied of these clock components have been the *Per* genes (*Per1*, *Per2*, and *Per3*), encoding mRNAs that both oscillate with a circadian rhythm, expressing high levels during the subjective day and low levels during the subjective night, and are photoinducible, with a phase dependence similar to that of light-induced phase shifts of behavioral rhythmicity. Initial studies of *Per1* and *Per2* mRNA (Shearman et al., 1997; Sun et al., 1997; Tei et al., 1997) and immunoreactive protein (Hastings et al., 1999; Field et al., 2000) suggested that expression occurred throughout the entire extent of the mouse SCN. This impression led to the conception of an idealized (linear) signal transduction pathway, in which axons of specialized retinal ganglion cells form the retino-hypothalamic tract (RHT) in the optic nerves and release glutamate at conventional synapses onto SCN "clock" cells. The resulting membrane depolarization leads to Ca^{2+} influx, CREB phosphorylation, and gene transcription, with the expression of proteins that reset the circadian pacemaker's core autoregulatory transcription-translation loop (for review, see Meijer and Schwartz, 2003).

But anatomical and physiological evidence from animals indicated that this simple concept lacked a critical *inter*-cellular dimension. Anatomically, only a subset of all rodent SCN cells are directly retinorecipient (for review, see Lee et al., 2003a). Physiologically, estimates of photoresponsiveness range from about one-fifth (by c-Fos immunoreactivity) (Castel et al., 1997) to about one third (by electrophysiology) located in the ventrolateral subdivision of the SCN (Meijer et al., 1986, 1998). No more than about 20% of the cells in this subset can be attributed to any one identified peptidergic phenotype (Romijn et al., 1996; Castel et al., 1997), and initial work suggested that no peptidergic cell type uniformly produced photosensitive responses.

Clues to solving this problem have been provided by using new markers for distinct SCN subregions and for individual SCN cells. In the hamster, calbindin-containing cells lying in the caudal core of the SCN were shown to be directly retinorecipient by tract-tracing and double-label electron microscopy (Bryant et al., 2000) and to virtually uniformly express immunoreactive Fos following a light pulse (Silver et al., 1996). Furthermore, light-induced *Per1* and *Per2* mRNAs were concentrated in this calbindin region (Hamada et al., 2001). Remarkably, endogenously rhythmic *Per1*, *Per2* and *Per3* expression was not detectable in this region but was observed instead in the dorsomedial SCN region marked by VP containing cells. Of note, electrophysiological recordings of identified calbindin cells in acutely-prepared hamster SCN slices have also demonstrated an absence of endogenous rhythmicity (Jobst and Allen, 2002).

A similar regional separation of rhythmic and non-rhythmic (but photoinducible) gene expression has been found for other genes, e.g., c-*fos* in rats and hamsters (Sumová et al., 1998; Guido et al., 1999ab; Schwartz et al., 2000), and for *Per* in other animals, e.g., rats (Yan et al., 1999; Yan and Okamura, 2002; Dardente et al., 2002). It had been thought that functional subdivisions in the mouse SCN were not so clearly segregated (King et al., 2003), but it was recently shown that *Per1* and *Per2* were light-induced but not detectably rhythmic in the GRP-containing cells of the mouse SCN (Karatsoreos et al., 2004). These results provide a clear example of functional segregation by SCN phenotype. Importantly, region-specific SCN rhythmicity extends beyond the genes; in electrophysiological studies *in vivo*, photically-responsive, neurons did not express the significant circadian rhythm in discharge rate that could be observed in photically-insensitive cells (Jiao et al., 1999; Saeb-Parsy and Dyball, 2003). Clearly, SCN neurons are not all functionally equivalent – light responsivity and endogenous rhythmicity of whole SCN tissue are based on a cellular division of labor.

Dissection of the SCN's Retinorecipient Subdivision

The two major peptidergic phenotypes that receive photic input via the RHT are VIP- and GRP-ergic neurons. Their mRNA and peptide levels exhibit oppositely-phased responses to light, with high levels of VIP during the dark and GRP during the light (Zoeller et al., 1992; Shinohara et al., 1993). In mice, GRP cells express *Per* genes following a light pulse but are not rhythmic in this response (Karatsoreos et al., 2004). In hamsters, calbindin delineates the region of light-induced *Per* expression and approximately 40% VIP and 60% GRP cells contain calbindin (LeSauter et al., 2002; Hamada et al., 2001). In rats, lateral (but not medial) VIP neurons in the ventral subdivision co-express GRP; the lateral (but not the medial) VIP cells receive retinal innervation and express photoinducible *Per1* (Kawamoto et al., 2003). Taken together, the data suggest an important role for GRP in photosensitivity. The intercellular mechanisms for coupling photoreceptive cells to and then resetting endogenously rhythmic cells are unknown, although a role for GRP is suggested. Intracerebroventricular injection of GRP during early night increased *Per* mRNA primarily in the *dorsal* mouse SCN, while the photic induction of *Per* was reduced in GRP receptor-deficient mutant mice, an effect also occurring primarily in the *dorsal* rather than in the ventral part of the nucleus (Aida et al., 2002). *Per1* and *Per2* expression in the dorsal SCN may be crucial to resetting the SCN by phase advances or delays (Yan and Silver, 2002).

The SCN's retinorecipient subdivision appears to play a critical role in the generation, and not just the entrainment, of circadian rhythmicity. Microlesions that ablated the calbindin region of the hamster SCN resulted in a loss of all measurable circadian rhythms, even though significant portions of the SCN with endogenously rhythmic *Per*-expressing cells survived the lesion (Lesauter and Silver, 1999; Kriegsfeld et al., 2004b). VIP and VPAC2 receptor knockout mice exhibit disrupted behavioral, molecular, and electrophysiological rhythmicity (Colwell et al., 2003; Piggins and Cutler, 2003). These kinds of data have stimulated a new model of SCN tissue organization, in which retinorecipient non-rhythmic “gate” cells provide resetting and synchronizing signals to individually rhythmic “clock” cells with different intrinsic periods (Antle et al., 2003). The gate provides daily input to the oscillators, and is in turn regulated (directly or indirectly) by the oscillator cells. Individual oscillators with initial random phases can self assemble so as to maintain cohesive rhythmic output. In this view, SCN circuits are important for self-sustained oscillation, and network properties distinguish the SCN from other tissues that lack resetting signals but rhythmically express clock genes.

Heterogeneity of Phase at Tissue and Single Cell Levels

Immunohistochemical detection of phosphorylated ERK/MAP kinase activity has demonstrated two distinct oscillations running in antiphase in different SCN regions (Obrietan et al., 1998; Coogan and Piggins, 2003; Lee et al., 2003b; Nakaya et al., 2003). During the

subjective day, pERK expression overlapped (but was not co-expressed) with that of VP (Lee et al., 2003b), while during the night, pERK expression was confined to a small region of “cap” cells (so named as they form a cap over the calbindin cells in the hamster SCN). This latter population of rhythmic cells behaved like a slave oscillator and was dependent upon the eye, even in conditions of constant darkness. Of note, within a central zone of the mouse SCN has been described a cluster of immunoreactive PER-expressing cells, in antiphase to the peak of PER expression at the end of the day (King et al., 2003); the relationship of these cells to the pERK region is unknown.

It is known that environmental lighting can dramatically alter regional phase relationships within SCN tissue. In the rat SCN, a sudden advance or delay of the LD cycle resulted in a transient desynchronization between the ventrolateral and dorsomedial subdivisions; ventrolateral gene expression shifted rapidly, while dorsomedial expression re-synchronized only gradually over days to weeks (Nagano et al., 2003). A stable, “forced” desynchronization of ventrolateral and dorsomedial subdivisions (again assessed by patterns of gene expression) has also been achieved by exposing rats to an artificially short 22-hr LD cycle (de la Iglesia et al., 2004). In this situation, the SCN was in a unique, reconfigured state; even though intercellular coupling *between* SCN subdivisions was lost, coupling *within* each subdivision was retained, suggesting that inter- and intradivisional synchronizing mechanisms may be different (for review, see Michel and Colwell, 2001). Perhaps the most dramatic example of functionally reconfigured SCN tissue is the phenomenon known as “splitting” in hamsters maintained in constant light, in which an animal's single daily bout of locomotor activity dissociates into two components that each free-run with different periods until they become stably coupled 180° (about 12 hr) apart. Splitting appears to be the consequence of a reorganized SCN with left and right halves oscillating in antiphase, as mRNAs characteristic of day and night are simultaneously expressed on opposite sides of the paired SCN (de la Iglesia et al., 2000).

Analysis of the SCN as an integrated tissue is now aided by powerful methods that make possible real time, simultaneous measurements of oscillatory gene activity over repeated cycles from multiple individual cells. As mentioned previously, bioluminescent rhythms in single neurons have been measured in tissue slices made from *Per1::luc* (Yamaguchi et al., 2003) and *Per1*::GFP (Quintero et al., 2003) transgenic mice. It has been shown that individual SCN cells in slices showed rather large phase differences in the peaks of their bioluminescent rhythms that persisted over repeated cycles (individual cellular periods were similar and stable). The phase order was not a stochastic property of the network, because it was restored in *Per1::luc* slices after cycloheximide was applied to first stop and then reset the cellular oscillations to the same initial phase. Moreover, intercellular phase differences were not an artifact of these transgenic preparations because similar differences have been demonstrated by electrophysiological methods in rat SCN slices (Schaap et al., 2003). In general, dorsomedial cells appeared to phase-lead (but did not appear to drive) ventrolateral ones in the *Per1::luc* slices (Yamaguchi et al., 2003), while a lateral-to-medial gradient was described in the *Per1*::GFP slices (Quintero et al., 2003) or none at all in electrical activity in the rat slices (Schaap et al., 2003). In the SCN harvested from hamsters sacrificed across the circadian cycle, the daily spread of gene expression was from dorsal to ventral (Hamada et al., 2004). What has become clear from all of these studies is that the duration of high molecular and electrophysiological activities of individual SCN cells appears to differ from the composite activities of the tissue as a whole (which generally lasts for most of the subjective day). The functional significance of heterogeneous cellular phases, as well as the mechanisms that keep the cells out of phase and direct their spatial organization, are not known. It is possible that their distribution and clustering can be configured by afferent input (Quintero et al., 2003). It could be that such plasticity of phase differences permits the encoding of a photoperiodic signal (Schaap et al., 2003).

Building a Global View: From Clock Genes to Circadian Behavior

Of course, it is the neural activity of the SCN in its proper context *in situ*, not gene expression in an isolated SCN *in vitro*, that regulates circadian behavior in whole organisms. Perhaps heterogeneous cellular phases might play a role as part of the temporal programming of SCN outputs (Kalsbeek and Buijs, 2002). Indeed, a recent single-unit electrophysiological study of the rat SCN *in vivo* has demonstrated that antidromically-identified SCN neurons innervating the arcuate or supraoptic nuclei express a very different firing rate rhythm (with peaks at the light-dark and dark-light transition phases) than do cells without such output projections (SaebParsy and Dyball, 2003). Also, anatomical tracing studies have indicated that there is a subset of SCN neurons that are both efferent to other hypothalamic nuclei and responsive to light (de la Iglesia and Schwartz, 2002; Munch et al., 2002); and in hamster, cells in the retinorecipient region delineated by calbindin and those in the rhythmic region delineated by VP both project to all of the same SCN target sites (Kriegsfeld et al., 2004a). These pathways provide a possible direct channel through the SCN for photic inputs to influence neural outputs. Such a route could underlie the immediate effect of light on the nocturnal rhythm of pineal melatonin secretion, in which light acts to acutely suppress nighttime melatonin production by an SCN pathway that is physiologically (Nelson and Takahashi, 1991) and pharmacologically (Paul et al., 2003) distinct from that mediating rhythm entrainment. Furthermore, cells from retinorecipient and from rhythmic regions of the SCN both project to all known targets of SCN neurons (Kriegsfeld et al., 2004a), providing another potential substrate for integration of photic and rhythmic information.

From Center to Network

The view of the SCN as a brain clock composed of 20,000 “clock” cells has been an extremely useful heuristic for advancing our knowledge of the molecular basis of circadian rhythmicity and for modeling formal properties of oscillators. At the same time, it has been remarkable to many students of the mammalian brain that the construct of a timing center has survived the experimental dissection of its component parts. The fact that the clock function of the SCN can be studied “in a dish” in acute slices or long-term cultures, in dispersed, dissociated cells and artificial cell lines likely accounts for its continuing heuristic value. It is hardly worth noting that the other once-popular brain centers, pleasure, satiety and hunger, did not share these easy tools of analysis. Nevertheless, the evidence is clear that specialization of function occurs within the SCN, and its network properties and signaling pathways are only starting to be revealed. Future approaches to understanding these properties – both electrophysiological (Pennartz et al., 1998) and genetic (Low-Zeddies and Takahashi, 2001) – will require regional and cellular levels of resolution.

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